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**Immunosurgery of Mouse Blastocyst**

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*Proc. Nat. Acad. Sci. USA*
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Immunology

# Immunosurgery of mouse blastocyst

**(antibody cytotoxicity/inner cell mass isolation/blastocyst permeability)**

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*Communicated by Clement L. Markert, October 1, 1975*

**ABSTRACT** **Mouse blastocysts with and without zonae pellucidae are susceptible to complement-dependent antibody cytotoxicity. Exposure of blastocysts to rabbit anti-mouse serum together with complement results in the death of all cells; however, when blastocysts are exposed to antiserum alone and then transferred to guinea pig complement, only the trophoblastic cells are killed. These results suggest that the mouse blastocyst is not permeable for certain antibodies. The inner cell masses can easily be separated from the remnants of trophoblastic cells and are then able to grow and differentiate *in vitro*. This method of immunosurgery can be used to obtain large quantities of pure inner cell masses in a relatively short period of time.**

The permeability of preimplantation mammalian embryos to macromolecules has primarily been studied in rabbits (1–3). Rabbit blastocysts possess a high degree of selective permeability depending on the type of macromolecule used and on the stage of development. Recently investigations of the mouse blastocyst (4) indicated that there is both a non-specific uptake of esterases into the mouse blastocoel and a selective uptake of maternal esterases into preimplantation mouse embryos. Since it is difficult, especially in mice, to distinguish between penetration of biochemical markers into the blastocoel and active uptake of these enzymes by trophoblastic cells, we decided to approach the question of permeability of mouse blastocysts using immunological methods.

We describe here a selective killing of trophoblastic cells using a two-step cytotoxicity procedure: preincubation with antiserum followed by separate exposure to complement. Aside from the important implications of these results to the study of the maternal-embryonic relationship, this technique allows for the ready collection and study of large numbers of pure inner cell mass cells, thus circumventing the complicated and time-consuming microsurgical methods.

## MATERIALS AND METHODS

**Embryos.** Virgin ICR, C3H/He, and C57BL/6 females were mated overnight, and checked for vaginal plugs the next morning; the day when the plug was observed was considered to be the first day of pregnancy. Blastocysts were flushed from uterine horns on the fourth day of pregnancy with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered Eagle's minimal essential medium (MEM) as described previously (5). Embryos were collected in the same medium, the zonae pellucidae were removed with Pronase (5), and the embryos were transferred to Eagle's MEM supplemented with 10% fetal calf serum, 10 mM glutamine, 100,000 units/liter penicillin, and 50 mg/liter streptomycin. Before exposure to antiserum, the embryos were incubated at 37° in 5% $CO_2$ in air for 3 hr.

**Antiserum and Complement.** Rabbit anti-mouse-spleen serum (kindly provided by Dr. David Aden, The Wistar Institute) was produced in a New Zealand White rabbit, which was bled 10 days after three intravenous injections of $4 \times 10^8$ ICR mouse spleen cells. Serum was heated at 56° for 30 min before use to inactivate rabbit complement. Fresh guinea pig serum was used as the source of complement at a final dilution of 1:16.

**Cytotoxicity Test.** Embryos were exposed to appropriate dilutions of antiserum and complement as indicated in *Results*. All dilutions were made with supplemented Eagle's MEM. Exposures were carried out in 50 μl droplets at 37° in 5% $CO_2$ in air. When embryos were exposed simultaneously to antiserum and complement, exposure time was usually 3 hr. In the two-step procedure, exposure to antiserum was 30 min (unless otherwise indicated), followed by three 5 min washings in MEM and a 30 min exposure to complement. The erythrosin B dye-exclusion method was used to determine the extent and localization of cell death (6).

**Growth of Embryos.** After exposure to antiserum and subsequent incubation with complement or after exposure to either antiserum or complement alone, the embryos were grown in supplemented Eagle's MEM in 60 mm Falcon plastic petri dishes at 37° in 5% $CO_2$ in air. The damaged trophoblastic layer was removed by pipetting the blastocysts through a small-bore glass pipette. All manipulations of embryos were carried out using a Nikon stereo microscope. Growing embryos were examined and photographed using a Wild M-40 inverted microscope with phase contrast optics.

## RESULTS

### Cytotoxicity

Blastocysts derived from all three strains of mice behaved equivalently in these experiments; hence, the results were pooled. Embryos exposed simultaneously to antiserum and complement for 3 hr displayed different distributions of dead cells depending on the dilution of antiserum. The percentage of embryos containing no living cells dropped sharply after antiserum dilution 1:96; the 50% end point dilution was between 1:100 and 1:200 (Fig. 1). Even at an antiserum dilution of 1:3072, however, more than 70% of the embryos contained no living trophoblastic cells.

When the embryos were exposed to antiserum, washed, and subsequently exposed to complement, all of the trophoblastic cells were destroyed in all dilutions up to 1:3072 (Fig. 1). Even at the lowest dilutions of antiserum, the cells of the inner cell mass were intact in all blastocysts examined. When these inner cell masses were cleared of remnants of the trophoblastic layer and transferred to either antiserum and complement or to antiserum and subsequently to complement, more than 80% of inner cell masses were totally destroyed in all dilutions up to 1:3052 (Fig. 1).

Abbreviation: MEM, minimal essential medium.

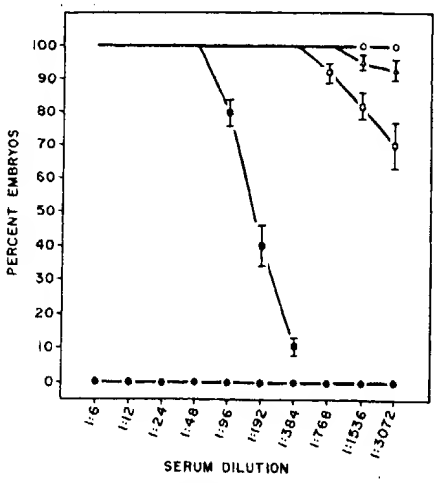


FIG. 1. Cytotoxicity of rabbit anti-mouse serum against mouse blastocyst. Embryos were exposed simultaneously to dilutions of rabbit anti-mouse serum and guinea pig complement (dilution 1:16) for 3 hr: blastocysts with all cells dead (■); blastocysts with only trophoblastic cells dead (□). Embryos exposed to dilutions of rabbit anti-mouse serum for 30 min, washed, and exposed to guinea pig complement (dilution 1:16) for 30 min: blastocysts with all cells dead (●); blastocysts with only trophoblastic cells dead (○). After the lysis of trophoblastic cells, inner cell masses were cleared of remnants of trophoblastic cells and exposed either to dilutions of rabbit anti-mouse serum and complement (dilution 1:16) simultaneously or in a two-step procedure; inner cell masses with all cells dead (△). The titration curves represent the results of seven separate experiments. Ten to 15 blastocysts or inner cell masses were used for each dilution in each experiment. Standard error is indicated by vertical bars. There was no cytotoxicity when blastocysts were exposed either to antiserum alone, or to complement alone, or to normal rabbit serum and complement simultaneously.

To further check the permeability of mouse blastocysts for antiserum, we exposed several groups of blastocysts to antiserum at a 1:100 dilution for 4, 10, 16, and 24 hr and then transferred them to complement for 30 min. Trophoblastic cells, in all cases, were completely destroyed while the inner cell masses remained intact. The inner cell masses were subsequently cleaned of trophoblastic cells and either exposed to antiserum and complement or grown *in vitro*. Those exposed to antibody and complement were lysed while those not exposed continue to grow and develop *in vitro* (see below). These results suggest that blastocysts are impermeable to antiserum even when exposed for 24 hr. Longer exposure is impractical as blastocysts begin to attach and differentiate after 24 hr so that the inner cell mass is no longer surrounded by the trophoblastic cells.

To test the effect of long exposure to complement, we exposed the blastocysts to antiserum at a dilution of 1:100 for 30 min and then transferred them to complement for a maximum of 6 days. After the initial destruction of trophoblastic cells, the inner cell masses (especially if cleaned from remnants of trophoblastic cells) continued to grow and differentiate.

## Morphology and development *in vitro*

Blastocysts exposed to antiserum appeared essentially unchanged in relation to control blastocysts (Fig. 2a). A few minutes after transfer to complement, the trophoblastic cells became swollen and began to lyse (Fig. 2b). At the same

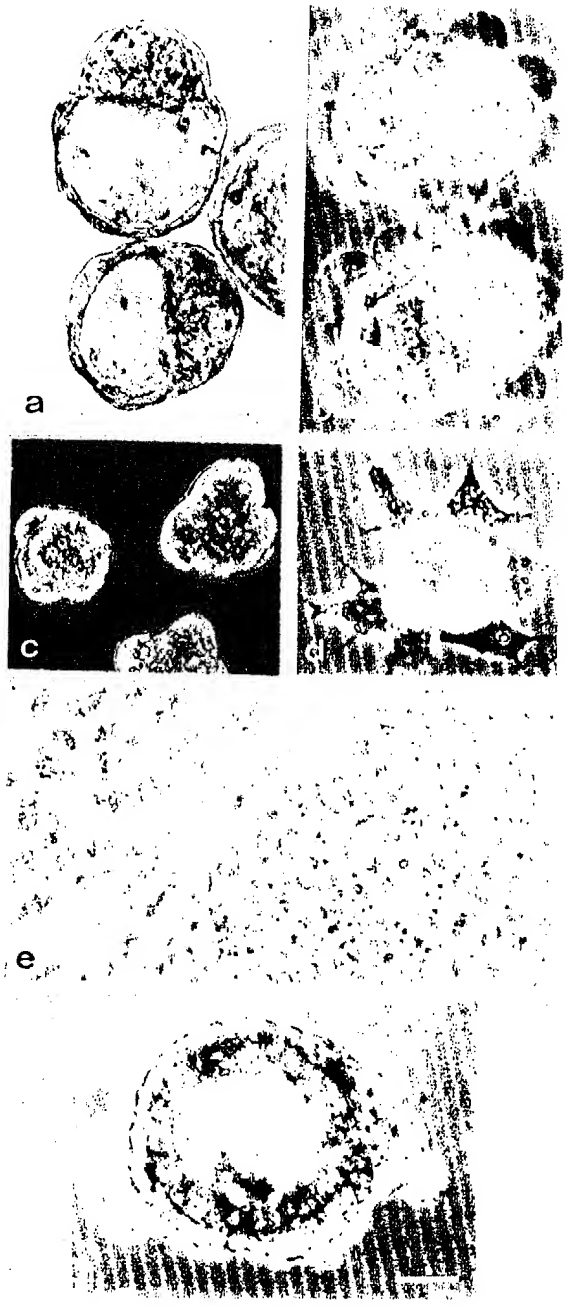


FIG. 2. Effect of two-step procedure on mouse blastocysts and their subsequent growth in culture. (a) Mouse blastocysts exposed to rabbit anti-mouse serum dilution 1:100 for 30 min; (b) blastocysts after exposure to antiserum and then to complement for 5 min—swelling of trophoblastic cells and demarcation of inner cell mass; (c) isolated inner cell masses cleared of dead trophoblastic cells; (d) inner cell mass after 24 hr in culture—attachment and outgrowth of cells containing vacuoles; (e) two of several cell types observed in culture of inner cell masses 6 days after plating—large cells with vacuolated cytoplasm and smaller cells with large nucleus and prominent nucleoli; (f) embryoid body derived from single inner cell mass after 4 days in culture—layer of outer cells and solid mass of cells inside. Scale bar = 30 μm in (f) applies to the entire figure.

time the inner cell mass, which in normal blastocysts appears continuous with trophoblastic cells (Fig. 2a), separated from the trophoblastic cells and was clearly visible within the blastocoel (Fig. 2b). When lysis of trophoblastic cells was complete and the inner cell masses were cleaned of dead trophoblastic cells by pipetting, the inner cell masses looked like small homogeneous cell clumps (Fig. 2c).

Inner cell masses plated in plastic dishes developed along two morphological routes: approximately half attached to the surface and from them relatively large polygonal cells with small vacuoles began to grow (Fig. 2d). Outgrowth of trophoblastic cells was never observed. The central mass of cells then either continued to grow as a solid mass and eventually developed into a structure resembling a 7-day-old mouse embryo, or it spread and formed a monolayer composed of several cell types (Fig. 2e). Half of the inner cell masses did not attach but continued to grow, floating in the medium, forming embryoid bodies (Fig. 2f) with a clearly visible outer cell layer and a solid mass of cells inside.

## DISCUSSION

There have been several reports on the permeability of the mouse blastocyst to macromolecules. Glass (7) reported the transfer of maternal serum antigens and of intravenously injected bovine serum albumin to cleaving mouse eggs and possibly to blastocysts. Sherman (4) also suggested the selective uptake of serum esterases by the cells of the mouse blastocyst as well as nonselective uptake into the blastocoel. Since the blastocoelic fluid was not analyzed separately (which would be technically very difficult in mice) these findings can only suggest that the mouse embryo shows selective uptake of macromolecules from surrounding fluids into the cytoplasm of trophoblastic and/or inner cell mass cells.

The barrier for nonselective uptake of large macromolecules probably consists of the trophoblastic cells themselves and the tight junctions between them. Tight junctions forming the zonula occludens are not present in early cleavage stages in the rabbit but appear in the blastocyst stage (8). Detailed analysis of junctional complexes between the trophoblastic cells of the mouse blastocyst revealed the presence of tight junctions and desmosomes (9). It has also been shown that neither ruthenium red nor concanavalin A can penetrate into the junctional complexes between the trophoblastic cells of the mouse blastocyst (10). These results would suggest that the mouse blastocyst is impermeable for molecules with a diameter of more than 40 Å, making the passage of the immunoglobulin molecule, with its considerably larger diameter (11), very unlikely.

Since the methods used in this study detect only the presence of active immunoglobulin molecules on the cell surface, the possibility that these molecules might enter the trophoblastic cells cannot be excluded. If they do, however, they are then either metabolized completely or rendered ineffective before entering the blastocoel fluid. Very similar results were obtained using rabbit anti-mouse cerebellar serum which was extensively absorbed with mouse tissues and reacted specifically with mouse brain cells, mouse sperm (12), and early mouse embryos (D. Solter and M. Schachner, manuscript in preparation). Our results therefore suggest that during a very short period in development, i.e., from the beginning of blastocyst formation to implantation, the cells of the embryo proper are efficiently shielded from any antibodies present in the uterine fluid by the trophoblastic cells. Since the zona pellucida does not prevent the penetration of antibodies (ref. 13, and our unpublished results) the cleavage stages prior to blastocyst formation are exposed to antibodies. When irreversible differentiation into embryonic and extraembryonic cells takes place during blastocyst formation, the impenetrability of the blastocyst to antibodies might be the first of the mechanisms which shield the mammalian embryo from immunological attack, securing its successful development.

The development *in vitro* of inner cell masses isolated immunosurgically can follow two pathways: attachment and cellular outgrowth or formation of embryoid bodies. From our results it is obvious that the trophoblast is not necessary for the attachment and outgrowth of the inner cell mass. The first cells present in outgrowth are highly vacuolated (Fig. 2c) and are probably equivalent to the cells of parietal entoderm; these and other cells observed in the outgrowth are not yet completely characterized. When the inner cell mass develops along the second pathway, the floating inner cell masses, after several days in culture, form structures composed of an inner homogeneous cellular core surrounded with a single layer of presumably entodermal cells (Fig. 2f) that resemble embryoid bodies observed in the culture of teratocarcinoma cells (14). The important difference between the isolated inner cell mass grown *in vitro* and the blastocyst *in vivo* is that the inner cell mass *in vitro* becomes completely surrounded with entodermal cells while the entoderm develops *in vivo* only on the side exposed to blastocoelic fluid. These results as well as those obtained with inner cell masses isolated microsurgically (15) suggest that the development of the entoderm might depend on the position of the cells in relation to their surrounding milieu. Our preliminary results (I. Damjanov and D. Solter, unpublished results) indicate that in the outer layer of the embryoid bodies derived from the inner cell masses there is a haphazard mixture of both visceral and parietal entodermal cells. The same observation was made when egg cylinders grown *in vitro* from intact blastocysts were examined ultrastructurally (16).

Besides having biological implication, these results demonstrate that this is an effective method for the isolation of large numbers of inner cell masses, making possible the study of their developmental, biochemical, and other properties. The procedure for isolating the inner cell masses is a counterpart to the method for obtaining trophoblastic vesicles by exposing early cleavage stages to tritiated thymidine and selectively killing the inner cell mass while allowing normal development of the trophoblast (17). It is now possible to dissect the mouse blastocyst efficiently into its component parts—trophoblast and inner cell mass—without using complicated and time-consuming microsurgery. This should greatly facilitate the investigations of the interaction between the trophoblast and the inner cell mass during development.

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